

Supplementary Information

RfIM mediates target specificity of the RcsCDB phosphorelay system for transcriptional repression of flagellar synthesis in *Salmonella enterica*

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Tab. S1. List of bacterial strains used in this study.

Strain	Genotype	Source or Reference
<i>Salmonella enterica</i> serovar Typhimurium LT2 strains		
TH437	WT	J. Roth
TH3466	F' 128 (pro-lac) <i>zzf382::Tn10dTc[del-20]</i> proAB47	Lab collection
TH9386	Δ <i>araBAD921::rflM</i> ⁺	J. Karlinsey
TH12883	Δ <i>araBAD996::rcsB</i> ⁺	Lab collection
TH12949	Δ <i>araBAD921::rflM</i> ⁺ <i>rcsB::MudJ</i>	Lab collection
TH13069	Δ <i>araBAD921::rflM</i> ⁺ <i>flhC5213::MudJ</i>	Lab collection
TH13654	Δ <i>araBAD1005::FRT rcsB::MudJ</i>	Lab collection
EM162	Δ <i>araBAD996::rcsB</i> ⁺ <i>rflM::MudJ</i>	This study
EM163	Δ <i>araBAD1005::FRT rflM::MudJ</i>	This study
EM169	Δ <i>araBAD1005::FRT rflM::MudJ</i> Δ <i>rcsDBC::FCF</i>	This study
EM221	Δ <i>araBAD1005::FCF flhC5213::MudJ rcsB::T-POP</i>	This study
EM222	Δ <i>araBAD996::rcsB</i> ⁺ <i>flhC5213::MudJ rflM2::T-POP</i>	This study
EM229	Δ <i>araBAD921::rflM</i> ⁺ <i>flhC5213::MudJ rcsB::T-POP</i>	This study
EM423	pNK2880 Δ <i>araBAD921::rflM</i> ⁺ <i>flhC5213::MudJ</i>	This study
EM477	Δ <i>araBAD1170::rflM(rcsB-HTH) flhC5213::MudJ</i>	This study
EM490	Δ <i>araBAD1170::rflM(rcsB-HTH) flhC5213::MudJ rcsB::T-POP</i>	This study
EM504	Δ <i>araBAD1005::FRT flhC5213::MudJ rflM2::T-POP</i>	This study
EM506	Δ <i>araBAD1171::rcsB(rflM-HTH) flhC5213::MudJ rflM2::T-POP</i>	This study
EM515	Δ <i>araBAD1005::FRT flhC5213::MudJ rflM2::T-POP</i> Δ <i>rcsDBC::FCF</i>	This study
EM517	Δ <i>araBAD1005::FRT flhC5213::MudJ</i>	Singer <i>et al.</i> 2014
EM521	Δ <i>araBAD996::rcsB</i> ⁺ <i>flhC5213::MudJ rflM2::T-POP</i> Δ <i>rcsDBC::FCF</i>	This study
EM541	Δ <i>araBAD1171::rcsB(rflM-HTH) flhC5213::MudJ rflM2::T-POP</i> Δ <i>rcsDBC::FCF</i>	This study

EM656	$\Delta araBAD1165::rflM(sdiA\text{-}HTH) flhC5213::MudJ$	This study
EM677	$\Delta araBAD1180::rcsB(sdiA\text{-}HTH) flhC5213::MudJ rflM2::T\text{-}POP$	This study
EM676	$\Delta araBAD1181::rflM(sdiA\text{-}HTH) flhC5213::MudJ rcsB::T\text{-}POP$	This study
EM704	$\Delta araBAD1005::FRT flhC5213::MudJ rcsB::T\text{-}POP$	This study
EM705	$\Delta araBAD1180::rcsB(sdiA\text{-}HTH) flhC5213::MudJ rflM2::T\text{-}POP$ $\Delta rcsDBC::FCF$	This study
EM706	$P_{flhDC}8093 (P_{flhDC}\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD1005::FRT$	Singer <i>et al.</i> 2014
EM707	$P_{flhDC}8124 (P_{flhDC} P1+ (-10 \text{ of } P2,P3,P4,P5,P6 \text{ changed to } GTTGGT)\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD1005::FRT$	Singer <i>et al.</i> 2014
EM711	$P_{flhDC}8128 (P_{flhDC} P5+ (-10 \text{ of } P1,P2,P3,P4,P6 \text{ changed to } GTTGGT)\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD1005::FRT$	Singer <i>et al.</i> 2014
EM808	$\Delta araBAD1005::FRT$	Lab collection
EM1055	$P_{flhDC}8093 (P_{flhDC}\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD921::rflM^{+}$	This study
EM1056	$P_{flhDC}8124 (P_{flhDC} P1+ (-10 \text{ of } P2,P3,P4,P5,P6 \text{ changed to } GTTGGT)\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD921::rflM^{+}$	This study
EM1060	$P_{flhDC}8128 (P_{flhDC} P5+ (-10 \text{ of } P1,P2,P3,P4,P6 \text{ changed to } GTTGGT)\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD921::rflM^{+}$	This study
EM1062	$P_{flhDC}8093 (P_{flhDC}\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD996::rcsB^{+}$	This study
EM1063	$P_{flhDC}8124 (P_{flhDC} P1+ (-10 \text{ of } P2,P3,P4,P5,P6 \text{ changed to } GTTGGT)\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD996::rcsB^{+}$	This study
EM1067	$P_{flhDC}8128 (P_{flhDC} P5+ (-10 \text{ of } P1,P2,P3,P4,P6 \text{ changed to } GTTGGT)\text{-}luxCDBAE\text{-}Km\text{-}P_{flhDC}^{+}) \Delta araBAD996::rcsB^{+}$	This study
EM1325	$\Delta rcsB$	This study
EM1432	$\Delta araBAD1005::FRT flhC5213::MudJ \Delta rcsB$	This study
EM1434	$\Delta araBAD1005::FRT flhC5213::MudJ rflM2::T\text{-}POP \Delta rcsB$	This study
EM1435	$\Delta araBAD996::rcsB^{+} flhC5213::MudJ rflM2::T\text{-}POP \Delta rcsB$	This study
EM1740	$rflM33::HA\text{-}FRT$	This study
EM1766	$\Delta araBAD1201::rflM^{+}\text{-}HA\text{-}FRT$	This study
EM2004	$RcsB_{D56E}$	This study
EM2005	$RcsB_{D56N}$	This study

EM2423	<i>ΔaraBAD1005::FRT flhC5213::MudJ ΔrflM RcsB_{D56E}</i>	This study
EM2424	<i>ΔaraBAD1005::FRT flhC5213::MudJ ΔrflM RcsB_{D56N}</i>	This study
EM2426	<i>ΔaraBAD921::rflM⁺ flhC5213::MudJ ΔrflM RcsB_{D56E}</i>	This study
EM2427	<i>ΔaraBAD921::rflM⁺ flhC5213::MudJ ΔrflM RcsB_{D56N}</i>	This study
EM2486	<i>ΔaraBAD921::rflM⁺ flhC5213::MudJ ΔrcsB</i>	This study
EM2504	<i>ΔaraBAD1201::rflM⁺-HA-FRT ΔrcsB</i>	This study
EM4358	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA</i>	This study
EM4365	<i>ΔaraBAD921::rflM⁺ ΔrcsB</i>	This study
EM4366	<i>ΔaraBAD996::rcsB⁺ ΔrflM33</i>	This study
EM4369	<i>ΔaraBAD921::rflM⁺ ΔlrhA</i>	This study
EM4370	<i>ΔaraBAD921::rflM⁺ ΔslyA</i>	This study
EM4372	<i>ΔaraBAD921::rflM⁺ ΔrtsB</i>	This study
EM4373	<i>ΔaraBAD921::rflM⁺ ΔfliZ</i>	This study
EM4420	<i>rflM33::HA-FRT ΔclpXP::FRT</i>	This study
EM4421	<i>rflM33::HA-FRT ΔclpX</i>	This study
EM4422	<i>rflM33::HA-FRT ΔclpA</i>	This study
EM4451	<i>rflM33::HA-FRT ΔclpXP-lon::FRT</i>	This study
EM4454	<i>ΔaraBAD1211::rcsB_{D56N} flhC5213::MudJ rflM2::T-POP ΔrcsB</i>	This study
EM4470	<i>ΔaraBAD921::rflM⁺ ΔprgH-hilD-hilA7791</i>	This study
EM4528	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA pSUMO-His₆-RflM</i>	This study
EM4529	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA pSUMO-His₆-RcsB</i>	This study
EM4530	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA pSUMO-His₆-RflM-RBS-RcsB</i>	This study
EM4570	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA pSUMO(-)</i>	This study
EM4586	<i>ΔaraBAD1201::rflM⁺-HA-FRT rcsB::T-POP</i>	This study
EM4598	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA ΔrcsB::tetRA pSUMO-His₆-RflM</i>	This study

EM4599	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA</i> <i>ΔrcsB::tetRA</i> pSUMO-His ₆ -RcsB	This study
EM4600	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA</i> <i>ΔrcsB::tetRA</i> pSUMO-His ₆ -RfIM-RBS-RcsB	This study
EM4601	<i>trp::(SpcR T7-RNAP lacO lacP lacI) flhC5213::MudA</i> <i>ΔrcsB::tetRA</i> pSUMO(-)	This study
EM4655	<i>rflM33::HA-FRT Δlon</i>	This study
EM4665	<i>ΔaraBAD1170::rflM(rcsB-HTH) flhC5213::MudJ ΔrcsB</i>	This study
EM4666	<i>ΔaraBAD1181::rflM(sdiA-HTH) flhC5213::MudJ ΔrcsB</i>	This study
EM4697	<i>ΔaraBAD1213::rflM(ΔHTH) flhC5213::MudJ</i>	This study
EM4711	<i>ΔaraBAD1213::rflM(ΔHTH) flhC5213::MudJ rcsB::T-POP</i>	This study
EM4727	<i>ΔaraBAD1213::rflM(ΔHTH) flhC5213::MudJ ΔrcsB</i>	This study
<hr/> <i>Escherichia coli</i> K12 strains <hr/>		
TH5314	BacterioMatch reporter strain, F' <i>P_{lacOR2-62} P_{lac}-bla-lacZ</i>	Stratagene
EM567	BacterioMatch pBT pTRG	This study
EM571	BacterioMatch pBT-RfIM pTRG-RfIM	This study
EM572	BacterioMatch pBT-RfIM pTRG-RcsB	This study
EM574	BacterioMatch pBT-RcsB pTRG-RcsB	This study
EM575	BacterioMatch pBT-RcsB pTRG-RfIM	This study
EM576	BacterioMatch pBT-LGF2 pTRG-Gal11P	This study
EM577	BacterioMatch pBT-RcsB pTRG-Gal11P	This study
EM578	BacterioMatch pBT-RfIM pTRG-Gal11P	This study
EM579	BacterioMatch pBT-LGF2 pTRG-RcsB	This study
EM580	BacterioMatch pBT-LGF2 pTRG-RfIM	This study
EM765	BL21(λDE3) pSUMO-His ₆ -RcsB	This study
EM1020	BL21(λDE3) pSUMO-His ₆ -RfIM-RBS-RcsB	This study
EM3064	BL21(λDE3) pSUMO-His ₆ -SUMO-RcsB _{D56E}	This study
EM4413	BL21(λDE3) pSUMO-His ₆ -SUMO-RfIM(ΔHTH)-RBS-RcsB	This study

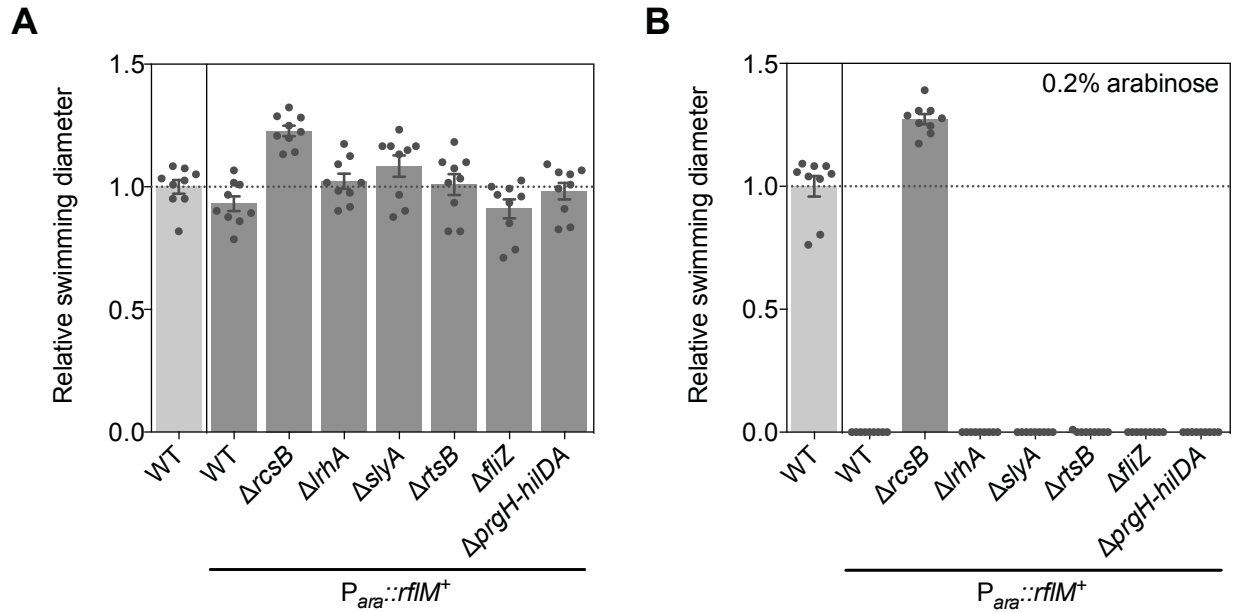
Supplemental Fig. S1.

Fig. S1. RfIM-mediated repression of motility does depend on the presence of RcsB, but not other *flhDC*-specific regulators.

Relative swimming diameters of deletion mutants of *flhDC*-specific regulators ($\Delta rcsB$, $\Delta lrhA$, $\Delta slyA$, $\Delta rtsB$, $\Delta fliZ$, $\Delta prgH-hilDA$) that harbored *rfIM* under control of an arabinose-inducible promoter ($P_{araBAD::rfIM}^+$) were analyzed under non-inducing (A) and inducing conditions (B). Swimming diameters were measured after incubation on 0.3% motility agar plates and normalized to the $P_{araBAD::FRT}$ wildtype control (WT). Bars represent mean values of 12 biological replicates and error bars represent the standard errors of the mean.

Supplemental Fig. S2.

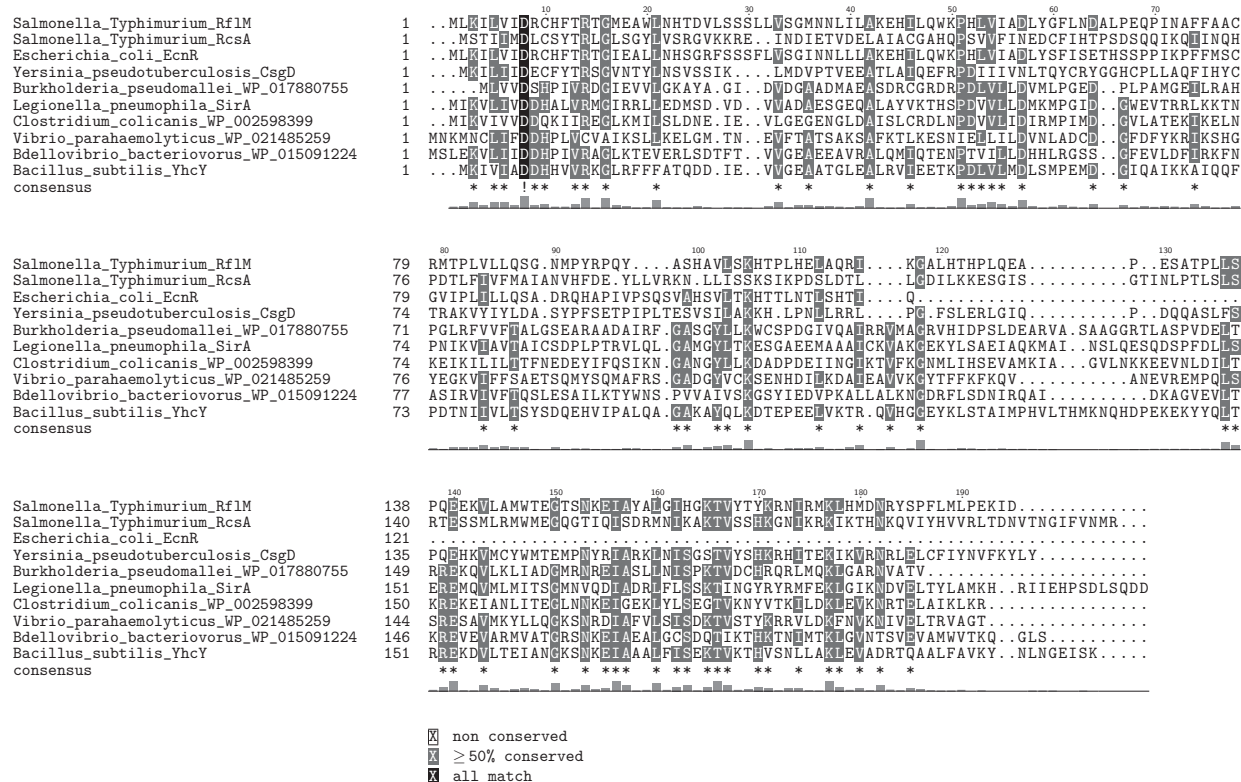
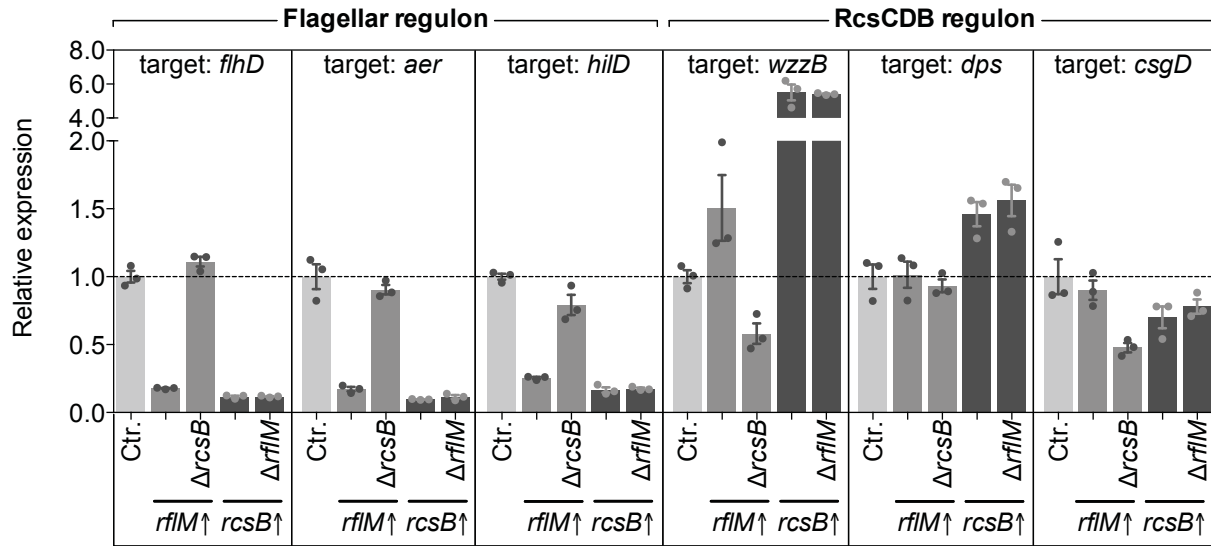
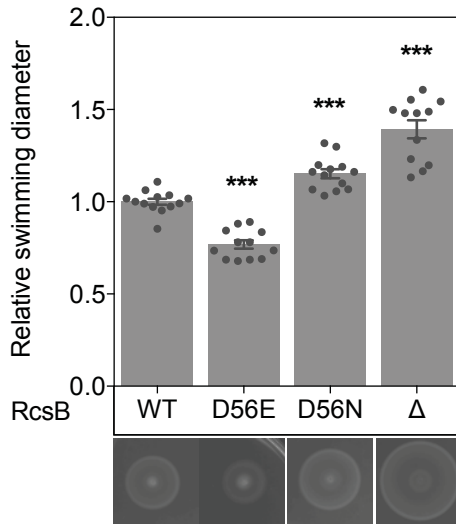


Fig. S2. Comparison of RfIM homologs by multiple sequence alignment.

Basic Local Alignment Search Tool (BLAST) was used to search for RfIM homologous sequences. The amino acid consensus sequence is shown at the bottom. Identical amino acids are shown with black shading marked with an exclamation mark (all match), whereas conserved amino acids are shown with grey shading marked with an asterisk ($\geq 50\%$ conserved). Bar heights below the consensus sequence indicate the degree of conservation.

Supplemental Fig. S3.**Fig. S3.** Relative transcript levels of flagellar and RcsCDB regulated genes.

Transcript levels of target genes belonging to the flagellar regulon (*flhD*, *aer* and *hilD*) and the RcsCDB regulon (*wzzB*, *dps*, *csgD*) were determined by qRT-PCR analysis. Analyzed strains include wildtype (Ctr.; EM808), *rflM* overexpressed from the P_{araBAD} promoter (*rflM*↑) in presence (TH9386) or absence of *rscB* ($\Delta rcsB$; EM4365) and *rscB* overexpressed from the P_{araBAD} promoter (*rscB*↑) in presence (TH12883) or absence of *rflM* ($\Delta rflM$; EM4366). Transcript levels were normalized against the reference genes *rpoB*, *gyrB* and *gmk*, and reported as relative expression compared to the wildtype control. Bars represent mean values of three independent biological replicates and error bars represent the standard errors of the mean.

Supplemental Fig. S4.**Fig. S4.** RcsB phosphorylation mutants are functional.

Relative swimming diameters of RcsB phosphorylation mutants RcsB_{D56E} (mimics phosphorylation; EM2004) and RcsB_{D56N} (cannot be phosphorylated; EM2005) compared to the wildtype control (RcsB_{WT}; TH437) and a *rscB* deletion mutant (Δ *rscB*; EM1325). Strains were incubated on motility plates containing 0.3% agar, swimming halo diameters were measured and normalized to the wildtype control. Bars represent mean values of eight biological replicates and error bars represent the standard errors of the mean. Exemplary images of the motility phenotypes are shown. Asterisks indicate significant difference to the WT control according to Student's t-test (*** $P < 0.0001$).

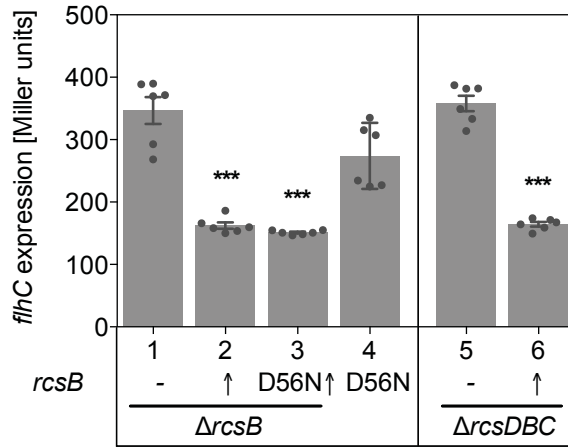
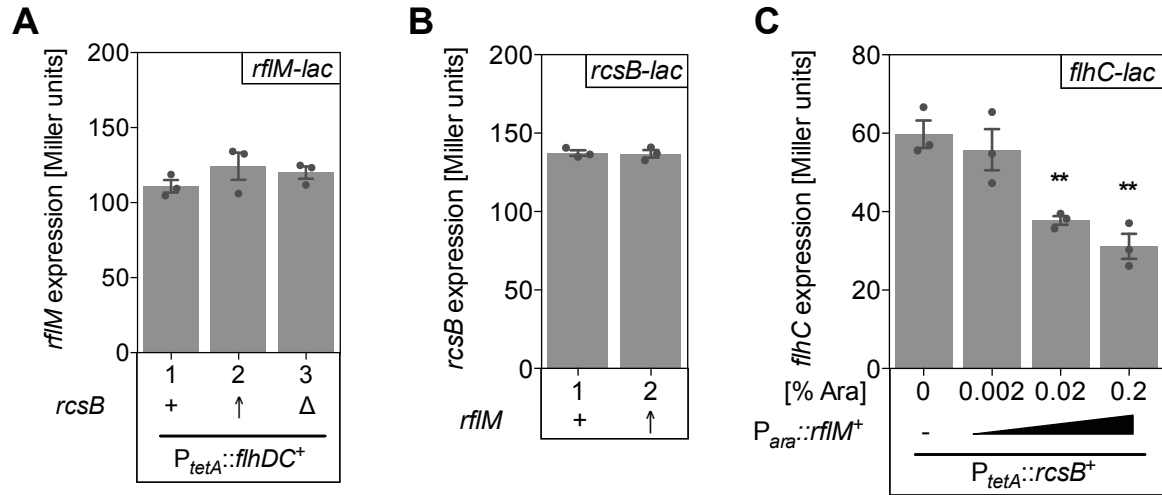
Supplemental Fig. S5.

Fig. S5. Overexpression of *rcsB* bypasses the requirement of RcsB phosphorylation for repression of *flhDC* operon expression.

Expression of a transcriptional *flhC-lac* fusion was analyzed upon P_{araBAD} -dependent *rcsB* overexpression (↑) and compared to a control strain (-). The *rcsB*_{D56N} mutation prevented RcsB phosphorylation. Strain backgrounds were either deleted for *rcsB* ($\Delta rcsB$) or for the RcsCDB phosphorelay cascade ($\Delta rcsDBC$) preventing phosphoryl group transfer to RcsB. Strains analyzed were EM1434 (1), EM1435 (2), EM4454 (3), EM4453 (4), EM515 (5) and EM521 (6). Bars represent mean values of six independent biological replicates and error bars represent the standard errors of the mean. Asterisks indicate significant difference to the corresponding control according to Student's t-test (*** $P < 0.0001$).

Supplemental Fig. S6.**Fig. S6.** RcsB and RflM do not regulate each other, but cooperatively repress *flhDC* expression.

A. Expression analysis of a transcriptional *rflM-lac* fusion under physiological *rcsB* expression level (+; EM163), *P_{araBAD}*-induced *rcsB* overexpression (↑; EM162) or in a RcsCDB deletion strain (Δ; EM169). All strains constitutively expressed *flhDC* from an ATc-inducible promoter (*P_{tetA}::flhDC⁺*) to ensure FlhD₄C₂-dependent *rflM* expression.

B. Expression analysis of a transcriptional *rcsB-lac* fusion under physiological expression level of *rflM* (+; TH13654) in comparison to *P_{araBAD}*-induced *rflM* overexpression (↑; TH12949).

C. Cooperativity of RcsB and RflM in repression of *flhDC*. *flhC-lac* levels were monitored under conditions of constant ATc-induced *rcsB* expression (*P_{tetA}-rcsB*) and additional titration of arabinose-induced *P_{araBAD}-rflM* (EM229) at the indicated arabinose concentrations [% Ara].

A-C. Bars represent mean values of three biological replicates. Individual data points are shown and error bars represent the standard errors of the mean. Asterisks indicate significant difference to the control according to Student's t-test (** *P*<0.005; *** *P*<0.0001).

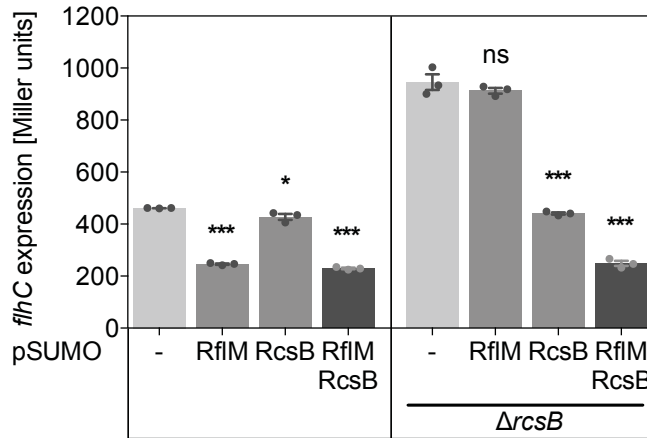
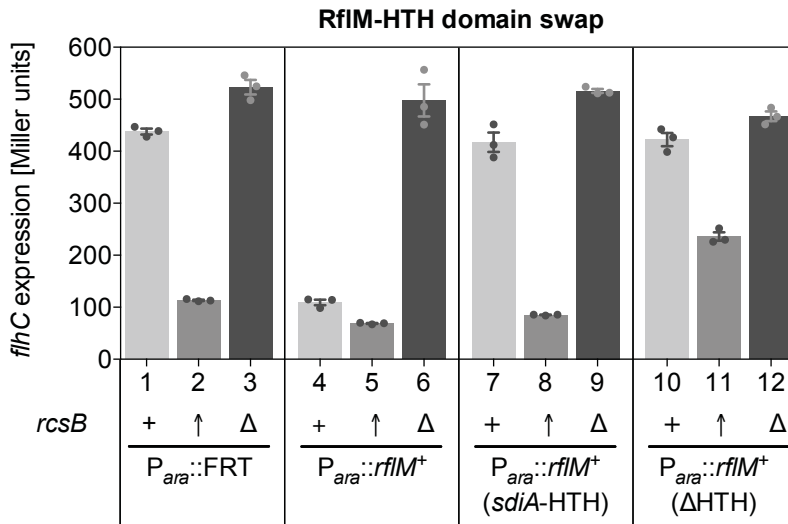
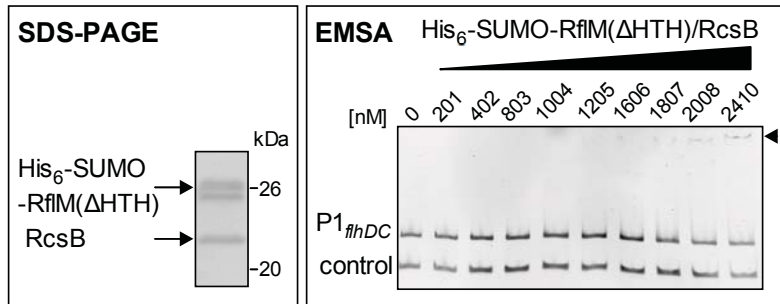
Supplemental Fig. S7.

Fig. S7. His₆-SUMO-RcsB and His₆-SUMO-RflM fusions used for protein purification are functional in repression of *flhC-lac* in *Salmonella enterica* strain LT2.

The activity of pSUMO-RflM, pSUMO-RcsB and pSUMO-RflM/RcsB fusion constructs in repression of *flhDC* was analyzed *in vivo* using a transcriptional *flhC-lac* fusion upon induction of *rcsB* or *rflM* overexpression with 0.2 mM IPTG. Induction of the pSUMO-RflM/RcsB construct resulted in co-expression of *rcsB* and *rflM*. The pSUMO empty plasmid (-) served as control. The *Salmonella enterica* reporter strains in the LT2 background constitutively expressed T7 RNA polymerase for expression of the RflM/RcsB constructs from the T7 promoter of the pSUMO plasmid and harbored a wildtype copy of *rcsB* or a deletion of *rcsB* ($\Delta rcsB$). Expression of *rflM* is FlhD₄C₂-dependent and the strains do not express *rflM* due to the transcriptional *flhC-lac* fusion. Bars represent mean values of three independent biological replicates and error bars represent the standard errors of the mean. Asterisks indicate significant difference to the corresponding control according to Student's t-test (* P<0.05, *** P<0.0001).

Supplemental Fig. S8.**A****B****Fig. S8.** The DNA-binding domain of RfIM is indispensable for repression of *flhDC*.

A. The helix-turn-helix (HTH) DNA-binding domain of RfIM was exchanged with the HTH domain of the LuxR-homolog SdiA of *Salmonella* ($P_{araBAD}::rfIM(sdiA-HTH)$) or deleted ($P_{araBAD}::rfIM(\Delta HTH)$) and the hybrid constructs were analyzed for their ability to repress *flhDC*. The wildtype *rfIM* control and the hybrid constructs were expressed from an arabinose-inducible promoter and the transcript levels of a *flhC-lac* fusion were determined in strains with physiological *rcsB* level (*rcsB*+), ATc-induced *rcsB* overexpression (*rcsB*↑) or *rcsB* deletion ($\Delta rcsB$). Strains analyzed were EM517 (1), EM704 (2), EM1432 (3), TH13069 (4), EM229 (5), EM2486 (6), EM656 (7), EM676 (8), EM4666 (9), EM4697 (10), EM4711 (11) and EM4727 (12).

B. Expression analysis and functionality test of purified RfIM protein deleted for its HTH domain in complex with RcsB (His₆-SUMO-RfIM(Δ HTH)/RcsB). Left: SDS-polyacrylamide gel electrophoresis of purified His₆-SUMO-RfIM(Δ HTH)/RcsB complex. Arrows indicate His₆-SUMO-RfIM(Δ HTH) (26 kDa) and presumably a degradation product of His₆-SUMO-RfIM(Δ HTH), as well as RcsB (24 kDa). Right: Electrophoretic mobility shift assay of the P1_{*flhDC*} promoter fragment (-271 to -71 relative to *flhD* ATG start site) with increasing amounts of purified His₆-SUMO-RfIM(Δ HTH)/RcsB complex as indicated. *gyrA* served as negative control DNA. The arrowhead indicates protein-DNA complexes.